



PATENT
Docket No. H 4086 PCT/US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Kropf et al. Art Unit: 1614
Appl. No.: 10/030,268 Examiner: Michelle Graffeo
Filed: March 19, 2002 Customer No.: 000055495
Title: COMPOSITE MATERIALS COMPRISED OF CALCIUM
COMPOUNDS AND PROTEIN CONSTITUENTS

DECLARATION UNDER 37 C.F.R § 1.132

I, Tilo Poth declare that:

1. I am a Senior Scientist, Project Manager at SusTech GmbH & Co. KG
Petersenstrasse 20, 64287 Darmstadt, Germany
2. I obtained a degree in Chemistry and Chemical Engineering in 1995 from
the Technical University Darmstadt, Germany (Technische Universität
Darmstadt, Germany and a Ph.D. degree in Chemistry in 1999 from the
Technical University Darmstadt, Germany (Technische Universität Darmstadt,
Germany.
3. I am familiar with the subject matter disclosed in the above-identified
patent application.
4. Under my supervision and control a study was carried out to assess the
ability of a novel protein-hydroxyapatite composite as described and claimed in
the above-identified application to induce biomineralisation in a tooth model. The

efficacy was tested versus a state of the art material (reference composite) as positive control described by Wang and coworkers.¹⁾

5. Tooth material, in particular dentin, is composed of inorganic (apatite) and organic (protein) parts that are intimately bound at the nanometer length scale forming complex composite materials. The process of their natural formation is labelled biomineralization. A variant of the dentin disk test was used to assess the biomineralizing ability of the composite claimed in the above-identified application. The test involves the microscopic investigation of mineralization processes on the surface of a dentine specimen. The mineralization can be considered bio analogous if newly formed material adapts both the morphology of the natural material and tightly adheres to it.

Dentin is distinguished by numerous channels (dentinal tubules) that run through the material having a width of 1-2 microns. To prepare adequate specimen, bovine dentin is cut and polished in such a way that the tubules end perpendicular at the surface. The mineralization processes observed during the treatment of the specimen with the composites leads to the formation of calcium phosphates on the dentin surface occluding the openings of the tubules either by loosely adhering crystals or by a compact layer. Therefore both the degree of tubular occlusion and the morphology of the formed material can be taken as a measure for the biomineralizing properties of the material employed.

6. The composite identified as "composite according to H04086" was prepared exactly as described in Example 2.1 of above-identified application. The composite identified as the "reference composite" was prepared exactly as described in the second paragraph of page 490 of Wang using Type I collagen from bovine tendons (Aldrich). In addition to the procedure of Wang, the collagen containing slurry was subjected to rotor stator mixing before ultra sonication to achieve the required dispersion.

¹⁾ R.Z. Wang, F.Z. Cui, H.B. Wen, C.L. Ma, H.D. Li, *Journal of Materials Science Letters*, 14, 1995, 490-492

7. Four (4) dentin specimens were prepared from bovine incisors. The polished slabs were then pre examined under an environmental electron microscope (ESEM) for open tubules. A particular area on the slabs was marked by engraving a fine square or triangle to later help the retrieval of the specific site. The ESEM method does not require the sputtering of the samples with a conductive layer of metal atoms. The method therefore allows repeated examinations and treatments of the same sample and therefore improves the statistic significance.

8. The four slabs were divided into two groups. Two slabs were treated with a composite according to H04086 and the remaining two slabs were treated with the reference composite.

Tab. 1 Dentin slabs and their treatment

Dentin Slab #	Treatment
1	Apatite Collagen Composite (Reference)
2	Apatite Collagen Composite (Reference)
3	Apatit Protein Composite According to H04086
4	Apatit Protein Composite According to H04086

For treatment, aqueous dispersions (2 wt %) of both composite materials were prepared. The slabs were immersed into the dispersions twice daily for ten minutes. Additionally the slabs were brushed for 1 minute daily prior to the first treatment with a soft bristle toothbrush to remove loosely adhering material. In between the slabs were stored in artificial saliva at 37°C to simulate conditions in the oral cavity. The artificial saliva emulates the mineral parts of natural saliva and is described in the literature²⁾. The treatment regimen was continued for 10

²⁾ J.M. ten Cate, M.J. Buijs, J.J.M. Damen, *Eur J Oral Sci*, 103, 1995, 362-367

days. After treatment the slabs were examined under the ESEM once more. Conditions before and after the treatment were compared.

9. The microscopic investigation shows that after the treatment cycle tubules are still clearly open when treated with the reference composite (Fig. 1 and Fig. 2).



Fig. 1 ESEM Micrograph of a dentin slab 1 before the treatment with the reference composite.

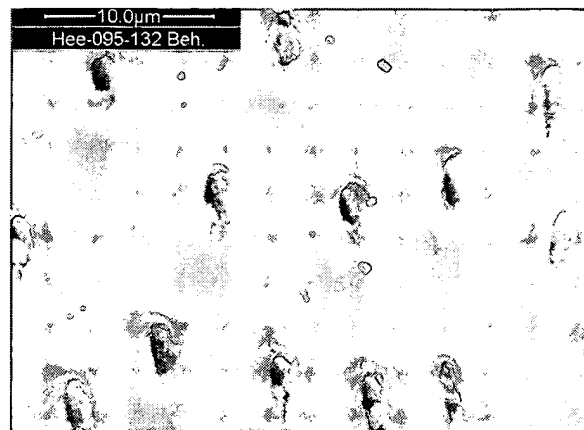


Fig. 2 ESEM Micrograph of dentin slab 1 after the treatment cycle with the reference composite

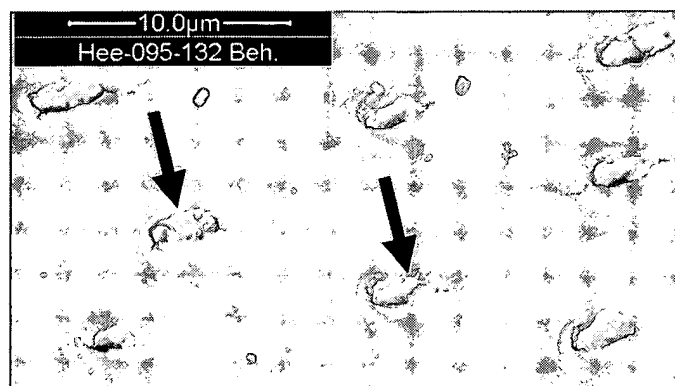


Fig. 3 Detailed view of the tubules of dentin slab 1 after the treatment with the reference composite. Some tubules are partly occluded by granular material

Further examination revealed that in some tubules small amounts of material were deposited (arrows in Fig. 3). The material however seems not to be connected to the surrounding dentin and is of granular morphology.

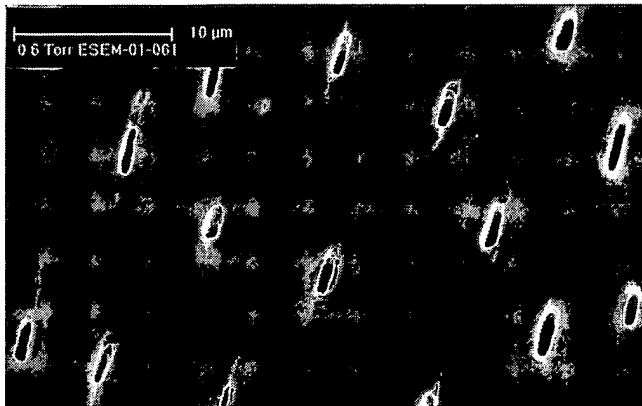


Fig. 4 ESEM Micrograph of a dentin slab 2 before the treatment with the reference composite.

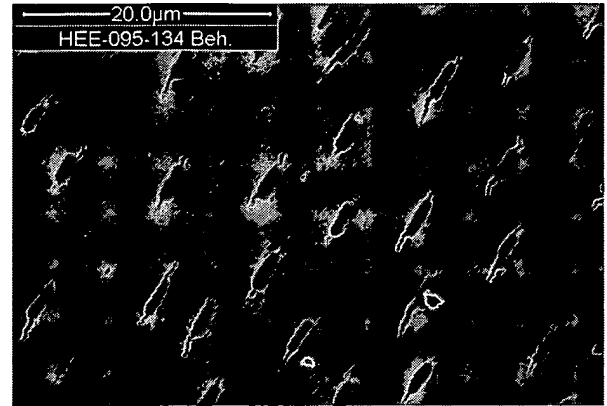


Fig. 5 ESEM Micrograph of dentin slab 2 after the treatment cycle with the reference composite

This finding applies also for the second dentin slab treated with the reference composite. Tubules here are a little narrower from the beginning but also remain clearly open.



Fig. 6 Detailed view of tubules of dentin slab 2 after the treatment cycle with the reference composite

In contrast, treatment with the apatite protein composite according to H04086 leads to a very effective mineralization which totally covers the tubules. The

material is smooth and intimately connected to the surrounding dentin (The cracks form as a consequence of the exposure to the vacuum and the electron beam). EDX analysis proves that it is calcium phosphate with a Ca/P ratio typical for apatite (Tab. 2).

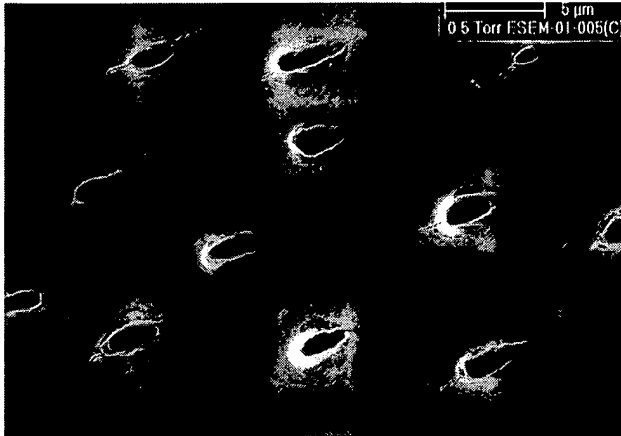


Fig. 7 ESEM Micrograph of a dentin slab 3 before the treatment with the composite according to H04086

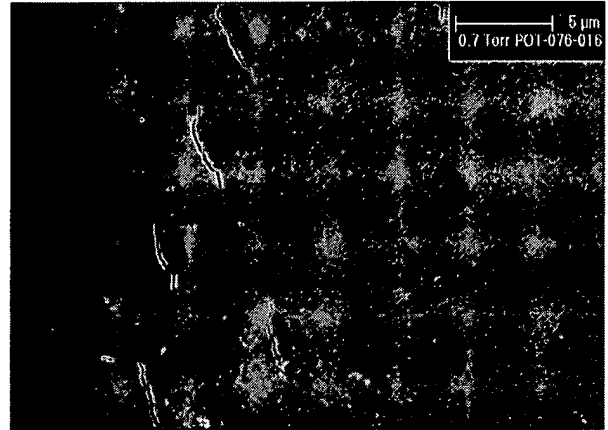


Fig. 8 ESEM Micrograph of dentin slab 3 after the treatment cycle with the composite according to H04086.

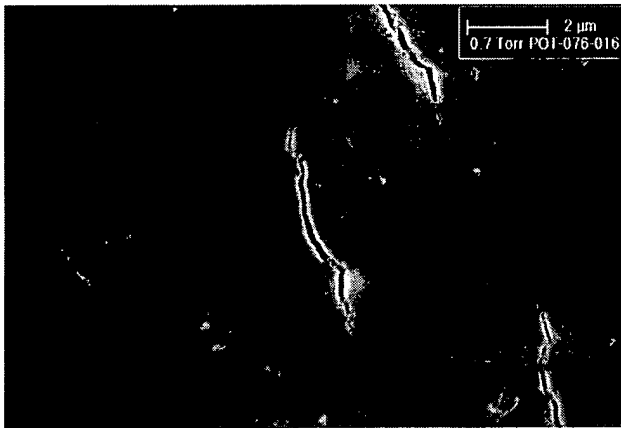


Fig. 9 Detailed view of tubules of dentin slab 3 after the treatment cycle with the reference composite according to H04086

Element	Wt%	At%
Ca	28,5	13,52
P	14,0	8,58
Ca/P	2,0 (2,16)	1,6 (1,7)
(theoret. apatite)		

Tab. 2 Typical contents of calcium and phosphate found by EDX analysis on closed tubules.

The results were confirmed by dentin slab 4. It should further be noted that the material which formed on the dentin surface does not possess the morphology of inorganic crystals but clearly resembles a natural composite material (Fig. 12)

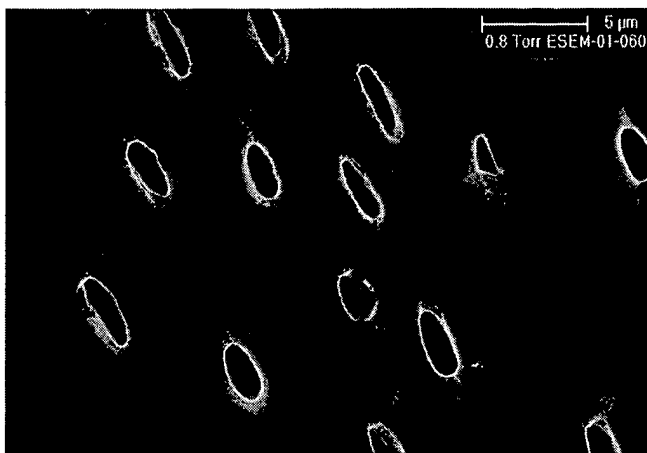


Fig. 10 ESEM Micrograph of a dentin slab 4 before the treatment with the composite according to H04086.

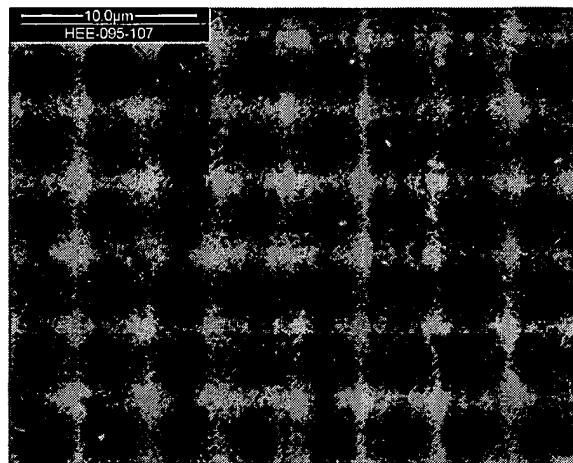


Fig. 11 ESEM Micrograph of dentin slab 3 after the treatment cycle with the composite according to H04086.

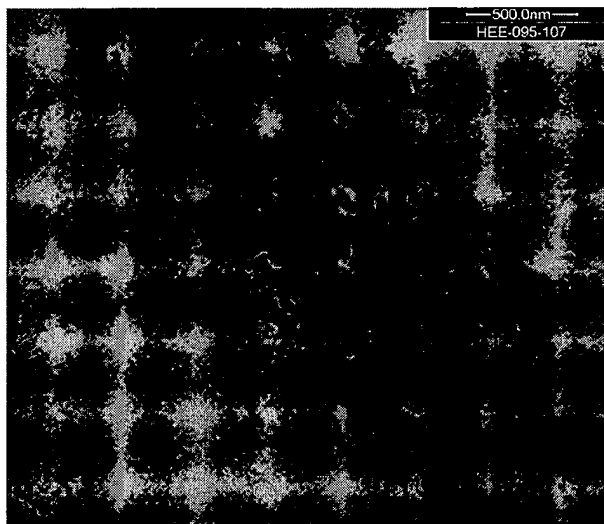


Fig. 12 Detailed view of tubules of dentin slab 3 after the treatment cycle with the composite according to H04086. It can be seen that the material does not exhibit the typical morphology of crystals but rather resembles a composite.

10. Further experimental evidence for the particular effective biomineralisation induced by the composite according to H04086 in contrast to the reference composite is illustrated by the pH-drop experiment³. Biomineralization involves the controlled formation of apatite according to Eq. 1 below. The associated release of protons causes a slight drop of pH in the environment of the

³ I) P. B. Messersmith, S. Starke *Chem. Mater.* 10, 1998, 117-124

mineralization. Consequently, measuring the pH over time yields a curve that by its slope gives a measure for the rate of mineralization and by its overall pH difference allows the assessment of the amount of apatite mineralized. For the experiment 100 µl of a 2% composite dispersion is injected into 30 ml of artificial saliva and kept at 37°C. The pH is monitored for 3 hours.

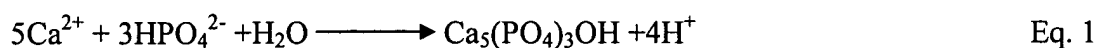


Fig. 13 shows two pH time traces. The dotted curve was recorded using the reference composite. The solid curve was acquired by using a composite according to H04086. It can clearly be seen that the composite according to H04086 nucleates apatite both at a much faster initial rate (greater slope) and to an greater overall extend (bigger pH drop after 3 hours) than the material of thereference composite. This provides further evidence that the composite according to the invention initiates and maintains biomineralisation processes more effectively.

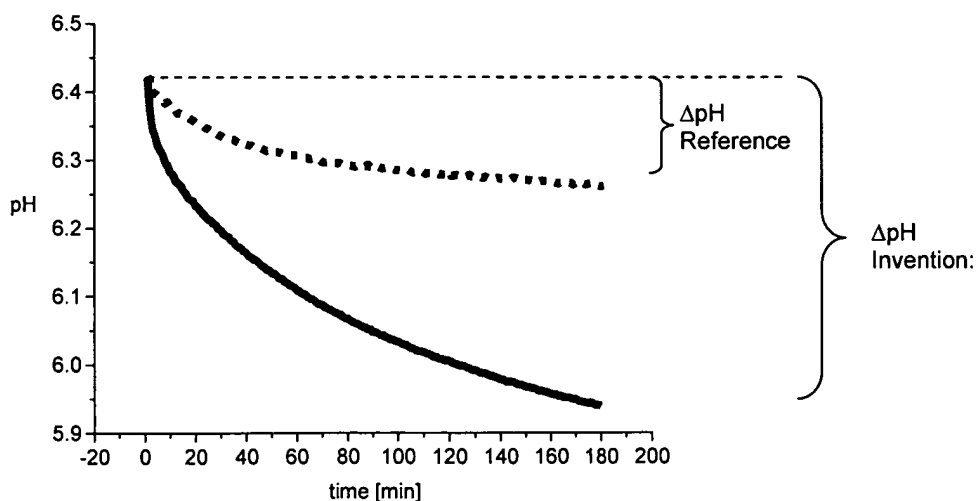
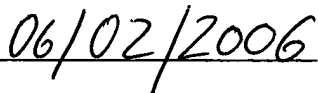


Fig. 13 pH time traces from a pH-drop experiment with composite material according to H04086 (solid curve) and reference composite (dotted curve).

11. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Tilo Poth



Date